REMARKS

Claims 1-3, 5-14, 16-24, 26-35 and 37-57 are pending. Claims 3, 8, 19, 29, and 40 have been withdrawn by the Examiner. Claims 3, 8, 10-14, 16-24, 26-33, 37-41, 43-45, 47-49, 51-53 and 55-57 have been newly canceled without prejudice. Claims 58-66 are newly added. Claims 1, 2, 5-7, 9, 34, 42 and 50 have been amended. Support for the amendments is found in the originally filed claims and the specification as discussed below. No new matter has been entered. Though many of the outstanding rejections apply to newly canceled claims, they are discussed below in the event the rejections are applied to the newly added claims, which contain essentially the same material as the newly canceled claims.

Claims rejection - 35 U.S.C. § 112, second paragraph

Claims 13, 14, 23, 24, 34, and 35 are rejected under 35 U.S.C. § 112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The office action indicates that the phrase "said drug" recited in Claims 13, 23, and 34 has no antecedent basis in claims 10, 21 and 31, respectively. Accordingly, Applicant has removed this language from the claims.

Reconsideration and withdrawal of the above ground of rejection is respectfully requested.

Claims rejection - 35 U.S.C. § 112, first paragraph – written description/new matter

Claims 1, 2, 5-7, 9-14, 16-18, 20-24, 26-28, 30-35, 37-39, and 41-57 are rejected under 35 U.S.C. § 112, first paragraph as containing subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s) had possession of the claimed invention at the time that the application was filed.

The office action indicates that the specification and claims as originally filed do not provide support for:

(A) the method of producing a fused cell product "for the reduction of the number of tumor cells in a patient" as recited in claims 1, 10 and 21;

(B) the method of producing a fused cell product "using PEG" as recited in claims 9, 20, 30 and 41; and

(C) the method of producing a fused cell product comprising "analyzing tumor associated antigens of said tumor sample" and "providing an established cell line comprising immortal human tumor cells having at least one tumor-associated antigen in common with said tumor sample" as recited in claim 31.

(A) Support for the claimed method of producing a fused cell product "for the reduction of the number of tumor cells in a patient" as recited in claims 1, 10 and 21 is found in the specification and the claims as originally filed.

Specifically, support for a method of producing a fused cell product "for the reduction of the number of tumor cells in a patient" as recited in instant claim 1, is found in originally filed claim 1 which recites in part, "A method for producing a plurality of dendritic cell/tumor cell hybrids which induce an anti-tumor response when applied to a patient causing a reduction of the number of tumor cells in said patient,..". .

Support for a method of producing a fused cell product "for the reduction of the number of tumor cells in a patient" as recited in instant claim 10, is found in originally filed claim 10 which recites in part, "A method for producing a dendritic cell/tumor cell hybridoma which induces an anti-tumor response when applied to a patient causing a reduction of the number of tumor cells in said patient,". However, claim 10 has been canceled without prejudice by Applicant, rendering the rejection moot.

Support for a method of producing a fused cell product "for the reduction of the number of tumor cells in a patient" as recited in instant claim 21, is found in originally filed claim 21 which recites in part, "A method for producing a dendritic cell/tumor cell hybridoma useful for the induction of an anti-tumor response when applied to a patient causing the reduction of the number of tumor cells in said patient,". However, claim 21 has been canceled without prejudice by Applicant, rendering the rejection moot.

Additional support for a method of producing a fused cell product "for the reduction of the number of tumor cells in a patient" as recited in the instant claims is disclosed throughout the instant specification for example at page 60, paragraphs 1 and 2, which disclose that injections of the hybrid cells "prevented the growth of pre-established P815 mastocytoma and provided long term protection." When mice were inoculated with a lethal dose of P815 and subsequently received intraperitoneal injections of hybrid cells, long term tumor protection resulted in 55% of the animals (see Figure 12). In the untreated animals, the tumors grew and killed the animals. The treated mice were also protected against a second tumor challenge (page 60-61, bridging

paragraph; Figure 13). More generic descriptive support is found on page 15, second full paragraph, of the present specification which discloses that "the term "activation of immune cells in vivo" which refers to the immune rejection of a residual tumor, as measured by its reduction in size and by the survival of the patient, as shown for mice in Example 5C or Example 12.

(B) Support for the claimed methods of producing a fused cell product "using PEG" as recited in claims 9, 20, 30 and 41 is found in the claims as originally filed and throughout the specification.

Specifically, support for the claimed method of producing a fused cell product "using PEG" as recited in instant claim 9 is found in originally filed claim 9 which recites "The method of claim 1 wherein the fusion in step (d) is carried out using PEG". Originally filed claim 1 is drawn to a method for producing a plurality of dendritic cell/tumor cell hybrids, with step (d) reciting: "fusing said dendritic cells with said tumor cells to produce a plurality of hybrids".

Support for the claimed method of producing a fused cell product "using PEG" as recited in instant claim 20 is found in originally filed claim 20 which recites "The method of claim 10 wherein the fusion in step (e) is carried out using PEG". Originally filed claim 10 is drawn to a method for producing a plurality of dendritic cell/tumor cell hybridomas, with step (e) reciting: "(e) fusing said dendritic cells with said immortal tumor cells to produce a plurality of hybridomas,". However, claim 20 has been canceled without prejudice by Applicant, rendering the rejection moot.

Support for the claimed method of producing a fused cell product "using PEG" as recited in instant claim 30 is found in originally filed claim 30, which recites "The method of claim 21 wherein the fusion in step (d) Is carried out using PEG". Originally filed claim 21 is drawn to a method for producing a plurality of dendritic cell/tumor cell hybridomas, with step (d) reciting: "(d) fusing said immortal dendritic cells with said tumor cells to produce a plurality of hybridomas,". However, claim 21 has been canceled without prejudice by Applicant, rendering the rejection moot.

Support for the claimed method of producing a fused cell product "using PEG" as recited in instant claim 41 is found in originally filed claim 41, which recites: "The method of claim 31 wherein the fusion in step (e) is carried out using PEG". Originally filed claim 31 is drawn to a method for producing a plurality of dendritic cell/tumor cell hybridomas, with step (e) reciting: "fusing said dendritic cells with said immortal tumor cells to produce a plurality of

hybridomas,". However, claim 41 has been canceled without prejudice by Applicant, rendering the rejection moot.

Additional support for the use of PEG in the claimed methods of producing a fused cell product is also found throughout the Examples disclosed in the instant specification, e.g., Example 3 (page 33, line 23), Example 9 (page 46, lines 21-25) and Example 12 (page 54, line 5).

(C) Support for the method of producing a fused cell product comprising "analyzing tumor associated antigens of said tumor sample" and "providing an established cell line comprising immortal human tumor cells having at least one tumor-associated antigen in common with said tumor sample" as recited in instant claim 31 can be found in originally filed claim 31 which recites in part; "A method for producing a dendritic cell/tumor cell hybridoma useful for the induction of an anti-tumor response, said method comprising: (a) providing a sample of a tumor against which said response is needed, (b) analyzing tumor-associated antigens of said tumor sample, (c) providing an established cell line comprising immortal human tumor cells having at least one tumor-associated antigen in common with said tumor sample,".

Though supported as described above, Applicant has cancelled without prejudice, the method step of "analyzing tumor associated antigens of said tumor sample" in instant claim 31, rendering the rejection of this phrase moot.

Additional support for the instantly claimed method encompassing the step of providing an established cell line comprising immortal human tumor cells having at least one tumor-associated antigen in common with said tumor sample is found in the specification at page 25, lines 8-12, which teaches that "as an alternative, a pre-established immortal human tumor cell line can be used, provided that at least one of the tumor-associated antigens from the patients' tumor cells are matched to these pre-established immortal tumor cell." See also Embodiments J, K, L, and M at pages 29-30 of the specification.

In light of the above claim amendments and remarks, Applicant respectfully requests reconsideration and withdrawal of the instant rejections.

Rejection under 35 U.S.C. § 112, first paragraph – written description/new matter

Claims 21-24, 26-31, 44 and 52 are rejected under 35 U.S.C. § 112, first paragraph as containing subject matter that was not described in the specification in such a way as to

reasonably convey to one skilled in the relevant art that the inventor(s) had possession of the claimed invention at the time that the application was filed.

The Office Action indicates that the specification and claims as originally filed do not provide support for Claims 21-24, 26-31, 44, and 52, drawn to a method of producing DC/tumor cell hybrids which comprises providing an immortal cell line comprising immortal autologous or HLA compatible or allogeneic DCs by isolation of DCs from bone marrow, lymph or blood or preparing said DCs by differentiating in vitro proliferating DC precursors isolated from bone marrow, lymph or blood. Specifically, the office action indicates that there is "no support for the immortal cell line DCs of the claim being isolated from these sources". The instant claims have been canceled by Applicant, rendering the rejection moot.

Support for the claimed methods of producing DC/tumor cell hybridomas which comprises providing an immortal cell line comprising immortal autologous or HLA compatible or allogeneic DCs derived from primary cultured DCs from bone marrow, lymph or blood is found throughout the specification, specifically in paragraphs 104, 105, and 108 of the instant specification as published on the USPTO web site as 20030031656 as follows:

[0104] "A sample is provided with a source of DLCs or DCs..."

[0105] "The DLCs or DCs are prepared for cell fusion, in one of the 4 following ways:"

[0108] "(3) Immortal DLCs or DCs can be derived from primary-cultured DLCs or DCs,".

Applicant has amended deleted the recitation of the term "proliferating" from the instant claims. Applicant traverses the inclusion of claim 31 since it does not recite an immortal dendritic cell line.

As described at page 26, lines 9-10, immortal DCs are derived from primary –cultured DCs which are in turn derived from blood, bone marrow, lymph or other tissue as described (e.g. see page 25, line 28-29; page 26, lines 1-2). The Examiner further argues that the cite at page 26 discloses the isolation of DCs for culture, not for fusion of step (d). In the section previously referred to (page 25, lines 13-25) it states that the sample which is used as the source of the DCs is "from the patient" (line 17) or "from a healthy, HLA-compatible donor" (lines 17-18). In the following paragraph, it states that the "DLCs or DCs are prepared for cell fusion in one of the 4 following ways". Clearly the DCs discussed in the preceding paragraph at lines 17-18 are intended for use in cell fusion contrary to the assertion of the Examiner.

The specification previously referred to at page 26, lines 1-18 teach primary cultured, immortal and drug-sensitive immortal DCs which are used for fusion with at tumor partner (page 26, lines 19-20). The sections at page 28, line 20 to page 30, line 13 describe embodiments A-M. Each section described that the DCs are fused with tumor cells (see specifically page 28, line 21, 30; page 29, lines 8, 17, 267-27; page 30, lines 5-6). Accordingly, it is respectfully submitted that the preparation of the DCs for subsequent fusion with tumor cells is described in the specification, at least at the sections discussed above.

In view of Applicants' amendments and arguments, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

Rejection under 35 U.S.C. § 103(a)

Claims 1, 5, *6,* 7, 9, 10, 11, 16, 17, 18, 20, 21, 22, 26, 27, 28, and 29 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Guo et al. (1994) in view of Sornasse et al. (1992) and Young, et al. (1990).

The office action states that the Guo reference:

"differs from the claimed invention only in that it does not teach the use of a DC as the antigen presenting component of the hybrid nor the isolation of said DCs from blood", page 5 of the instant office action",

and that:

"It would have been prima facie obvious to one of ordinary skill at the time the invention was made to produce the hybrids/hybridomas of Guo et al., by the method of Guo et al., substituting a DC for the B cell in said hybrids/hybridomas, as taught by Sornasse et al, said DCs being isolated from human blood, as taught by Young et al. On of ordinary skill in the art at the time of the invention would have been motivated to make the substitution because, while both B cells and DCs are capable of inducing IL2 secretion in vitro, DCs induce a more vigorous response , including a TH1 response, in vivo, as taught by Sornasse et al. 'Our data emphasize the main role of DC in initiating primary responses in vivo' ", page 5 of the instant office action, emphasis added.

Although no explicit teaching, suggestion, or motivation is required in order to combine the teachings of the references, a determination of obviousness must be made based on what a person of ordinary skill in the pertinent art would have known at the time of filing based on Graham inquiries concerning the scope and content of the prior art; the differences between the claimed invention and the art; and the level of ordinary skill in the art. The newly published guidelines for determining obviousness after KSR (Federal Register, Vol. 72, No. 195; October 10, 2007) provide a number of rationales that can be used to support a legal conclusion of obviousness. Each is discussed herein. None of the rationales provide support an obviousness rejection based on the cited art.

Applicant contends that at the time of filing, one skilled in the art would not have been motivated to produce a plurality of dendritic cell/tumor cell hybrids which induce an anti-tumor response when applied to a patient causing a reduction of the number of tumor cells in said patient, *said method comprising fusing said dendritic cells or dendritic cell precursors from bone marrow, lymph or blood with said tumor cells,* where accordingly, the dendritic cell is not a T-lymphocyte or B-lymphocyte. As discussed in the instant specification in paragraph 0011 of the published application on the USPTO's website, Guo et al. fused a rat hepatoma cell line *with in vivo activated B-lymphocytes,* and showed that some of the resulting B-cell/tumor cell hybridomas induced tumor-resistance in syngeneic rats and also cured the animals of a small pre-established tumor. However, as discussed in the specification in paragraph 0011, Guo's method, is generally inapplicable to human patients, because of the difficulty in obtaining the *in vivo* activated B cells from human spleens for use as fusion partners, and also because Guo's method involves the administration of the soluble tumor antigen in complete Freund's adjuvant to prime the B cells used as a partner fusion, a technique which cannot be used in humans.

Surprisingly, Applicant discovered the instantly claimed methods of producing dendritic cell/tumor cell hybrids from *nonactivated* antigen presenting cells (as opposed to the B cell/tumor cell hybrids taught by Guo et al. which were produced using *activated B* cells) that are able to induce an anti-tumor response when administered to a patient. Specifically, the *novel dendritic cell/tumor cell hybrids* produced by the instantly claimed methods maintain the physiological activities of presenting endogenous antigen to antigen specific naive T cells in vivo, resulting in their activation and subsequent induction of an anti-tumor immune response. That these novel dendritic cell/tumor cell hybrids maintain both *the physiological attributes* of

their starting dendritic cells *and the tumor associated antigens of their tumor fusion partner* is unpredictable, even according to the Examiner's standards.

In related co-pending application 09/951,849, of which this instant application is a divisional, the same Examiner refers to MPEP 2164.03, and states that:

"The MPEP further states that physiological activity can be considered inherently unpredictable" , (emphasis added) (page 6 of office action mailed April 25, 2005). Further, the physiology of antigen presentation to T cells in B cell blasts versus dendritic cells is still not established in 2007 as evidenced by the following excerpt from the first paragraph of an article published in PNAS in January of 2007:

> "The initiation of adaptive immunity requires presentation of antigens by dendritic cells (DCs) to naive T lymphocytes (1). Why DCs are more efficient at stimulating naive T cells than other antigen-presenting cells (APCs) remains incompletely understood. DC specializations that may contribute include the expression of high surface levels of MHC class II (MHC II) and costimulatory molecules, their regulated patterns of maturation and migration that coordinate their abilities to present antigens acquired in the periphery after arrival in lymphoid organs, their elaboration of long membrane projections that facilitate T cell contacts, and their secretion of an array of immunostimulatory cytokines (2). It seems likely, however, that other features remain to be discovered." (emphasis added) (Unternaehrer et al. Proc Natl Acad Sci U S A . 2007 January 2; 104(1): 234-239.)

Five of the seven published rationales that can be used to support a legal conclusion of obviousness, outlined below, require predictability.

Rationale A. Combining prior art elements according to known methods to *yield predictable* results.

Rationale B. Simple substitution of one known element for another to *obtain predictable results.*

Rationale C. Use of a known technique to improve similar devices (methods or products) in the same way.

Rationale D. Applying a known technique to a known device ready for improvement to *yield predictable results.*

Rationale E. Obvious to try—choosing from a finite number of identified, *predictable solutions,* with a reasonable expectation of success.

Rationale F. Known work in one field of endeavor may prompt variations of it for either use in the same field or a different one based on design incentives or other market forces *if the variation would have been predictable to one of ordinary skill in the art.*

Rationale G. Some teaching, suggestion, or motivation in the prior art that would have lead one of ordinary skill to modify the prior art reference or combine prior art references to arrive at the claimed invention. Despite the Examiner's acknowledgement of the inherent unpredictability of physiological activity, the Examiner concludes in the instant office action that the ordinary skilled artisan would expect the dendritic cell hybrid to retain the antigen presenting cell (APC) characteristics of the dendritic cell, by stating that:

> "Guo teaches that the B cell hybrids retain the characteristics of the APC fusion partner, e.g., the B cell. Thus, the ordinarily skilled artisan would expect the DC hybrid to retain the *APC characteristics* of the DC as well", (emphasis added) page 6 of the instant office action

Applicant respectfully traverses the Examiner's conclusion that the ordinarily skilled artisan would expect the Dendritic Cell/tumor hybrid to retain the *APC characteristics* of the Dendritic Cell (DC) based on the results of a B cell/tumor hybrids taught by Guo because the 2007 postfiling PNAS article referenced above teaches that the plasma membrane of B cell blasts and dendritic cells are different. Specifically the PNAS article teaches :

> "We compared DCs to B blasts to seek differences possibly responsible for DCs' superior antigen presentation ability. As expected, DCs express higher numbers of MHC II and costimulatory molecules than B blasts. It is surprising, however, that B blasts, known to be less efficient at T cell stimulation, actually achieve a higher density of MHC II molecules per unit of PM. These results are consistent with earlier findings that B cells require 10-fold more MHC-peptide complexes than DCs to effect the same level of T cell stimulation (37). Thus, although DCs are able to deliver a more potent stimulus to T cells, they do so while expressing a lower surface density of TCR ligand. Although this may in part be explained by DCs' higher density of CD86 and CD80, we propose that surface organization of MHC II may also play a role in facilitating antigen presentation. The patchy distribution of DC surface molecules presents the T cell with preclustered "packets" of MHC III-A and I-E, potentiating T cell activation by facilitating TCR clustering and signaling (7, 38). The DC is thus able to distribute TCR ligands over a larger surface area, allowing for simultaneous interaction with multiple T cells."

In view of the salient physiological and physical differences noted between B cell blasts and dendritic cells, Applicant does not agree that the ordinarily skilled artisan would expect the Dendritic Cell/Tumor hybrid to retain the APC characteristics of the Dendritic Cell.

Further in view of the salient physical and physiological differences between B cell blasts and dendritic cells, and in view of the fact that at the time of the invention, to Applicant's knowledge no dendritic cell/tumor hybrids had been reported as retaining the physiological activity of the dendritic cell required by the instant claims, indicates that the ordinary skilled artisan could not predict that the dendritic cell/tumor hybrid produced by the instantly claimed methods would retain the recited antigen presenting cell physiological activities attributed to the dendritic cell. *The inherent unpredictability as to the physiological phenotype of the dendritic cell/tumor hybrid therefore, negates the use of rationales A, B, D, E, and F listed above to support an obviousness rejection based on the cited art.*

Nor can Rationale "C", listed above as the use of a known technique to improve similar devices (methods or products) in the same way, be used to support an obviousness rejection based on the cited art. Though techniques of cell fusion of a tumor to a B cell were known in the art, as discussed above, there was no report to Applicant's knowledge of a method which successfully produced a dendritic cell/tumor hybrid at the time of the invention that was taught to possess the instantly claimed physiological characteristics, also discussed above. Further, a B cell is not at all similar to a dendritic cell, being dissimilar structurally, physiologically and with respect to localization within an individual as discussed above. Physiologically, unlike dendritic cells, a B cell is known for its specialized antibody producing capabilities, and also unlike dendritic cells, the B cell must be primed in order for it to stimulate naive antigen specific T cells with its processed endogenous antigen. Because of the physiological and structural dissimilarity of B cells and dendritic cells, a dendritic cell can not be substituted for an activated B cell in a fusion to a tumor cell in the method of Guo under this rationale to arrive at the dendritic cell/tumor cell hybrids encompassed by the claimed invention.

The final Rationale listed above, for supporting an obviousness rejection based on cited art does not suffice either. Rationale "G" requires some teaching, suggestion, or motivation in the prior art that would have lead one of ordinary skill to modify the prior art reference or combine the prior art references to arrive at the claimed invention. Sornasse et al.'s teaching that their data that emphasizes "the main role of DC in initiating primary responses in vivo", does not provide sufficient motivation for the claimed process since Sornasse et al.'s data encompasses dendritic cells that were pulsed with exogenous antigen, as opposed to the instant invention in which the dendritic cell/tumor hybrids have the physiological ability to process and

present to naive T cells tumor antigens which are endogenous to the hybrid cell. Thus, the stated motivation of the rejection, that dendritic cells "induce a more vigorous response, including a Thl response, in vivo", does not specifically teach, suggest or motivate one to use fusion. methods to produce a dendritic cell/tumor hybrid with the instantly claimed physiological capabilities, which does not require loading of exogenous peptides.

Claims 10, 11, 16, 17, 18, 20, 21, 22, 26, 27, 28, and 29 have been canceled by Applicant without prejudice, rendering the rejection of these claims moot.

In view of Applicants' amendments and arguments, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

Claims 2, 12, 33, 42, 43, 44, 46, 47, and 48 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Guo, et al. (1994) in view of Sornasse, et al. (1992) and Young, et al. (1990) as applied to Claims 1, 5, 6, 7, 9, 10, 11, 16, 17, 18, 20, 21, 22, 26, 27, 28 and 29 above, and further in view of US Patent No. 5,851,756.

Since claims 2, 12, 33, 42, 43, 44, 46, 47, and 48 depend ultimately from claims 1, 10, 21, or 31, which are neither taught nor suggested by the cited references as discussed above, the invention defined in claim 2, 12, 33, 42, 43, 44, 46, 47, and 48 is also patentably distinct from the teachings of the cited references, alone or in combination. Claims 12, 33, 43, 44, 47, and 48 have been canceled by Applicant without prejudice, rendering the rejection of these claims moot.

Claims 50-52 and 54-56 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Guo, et al. (1994) in view of Sornasse, et al. (1992) and Young, et al. (1990) as applied to Claims 1, 5, 6, 7, 9, 10, 11, 15, 16, 17, 18, 20, 21, 22, 25, 26, 27, 28 and 29 above, and further in view of US 5,637,483.

U.S. patent 5,637,483 was cited by the Examiner to show the use of irradiation of tumor cells in an anti-tumor vaccine to prevent proliferation of the tumor cells in the patient. Irradiation of tumor cells was known at the time of the claimed invention. However, since claims 50-52 and 54-56 depend from claim 1,10, and 21, which are neither taught nor suggested by Guo et al in view of Sornasse, et al. as discussed above, the invention defined in claims 50-52 and 54-56 is also patentably distinguished from the references, alone or in

combination. Claims 51-52 and 55-56 have been canceled by Applicant without prejudice, rendering the rejection of these claims moot.

Claims 13, 14, 23, and 24 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Guo, et al. (1994) in view of Sornasse, et al. (1992) and Young, et al. (1990) as applied to Claims 1, 5, 6, 7, 9, 10, 11, 16, 17, 18, 20, 21, 22, 26, 27, 28 and 29 above, and further in view of Reid, et al.

Reid, et al. was cited to show the use of HAT medium for killing of unfused tumor cells. This technique was known at the time of the claimed invention. However, since claims 13, 14, 23, and 24 depend from claim 10 and 21, which are neither taught nor suggested by Guo et al in view of Sornasse, et al. as discussed above, the invention defined in claims 13, 14, 23, and 24 is also patentably distinct from the references, alone or in combination. Further, Claims 13, 14, 23, and 24 have been canceled by Applicant without prejudice, rendering the rejection of these claims moot.

Claims Rejection - 35 *U.S.C. 102(b)*

Claims 1, 7, 9, 10, 13, 14, 18, and 20 are rejected under 35 U.S.C. § 102 (b) as being clearly anticipated by Breel, et al.

Applicant respectfully traverse the rejection of the instant claims, on the grounds that the hybrids taught by Breel do not meet all the limitations of the instantly claimed dendritic cell/tumor cell hybrids.

Breel et al. teaches methods of producing murine hybrid cell lines by selecting hybrid cells resulting from the fusion of a population of lymph cells enriched for dendritic cells to SP2/0 myeloma tumor cells, page 170. The hybrid cells were selected for their expression of the dendritic cell surface NLDC-145 marker. The objective was to produce a dendritic-like cell line as a convenient source of dendritic cells to be used as antigen presenting cells. Breel et al. teaches that the four selected hybrid cell lines expressed MHC Class II antigens characteristic of dendritic cells, but that the hybrids displayed no dendritic processes. Breel

et al. teaches that the four hybrids, when pulsed with an exogenous antigen, (KLH), can present this non tumor antigen to keyhole limpet hemocyanin (KLH) primed B cells. However, Breel et al. does not teach that the four hybrids have the physiological ability to induce an anti-tumor response when provided to a patient as required by the instant claims, an ability which encompasses processing and presenting to naive T cells tumor antigens which are endogenous to the hybrid cell. For this to be the case, the hybrids of Breel would still need to be loaded with antigens specific of the target tumor or be fused again with cancer cells of the target tumor, as thought by the present invention.

However, the Examiner contends that this required limitation is an inherent property of the referenced hybrids taught by Breel et al. Specifically, the office action states:

> "The recitation of producing...cell hybrids which induce an anti-tumor response when provided to a patient causing a reduction of the number of tumor cells in said patient is considered to be an inherent property of the hybrids", page 10 of the instant office action.

However, as discussed above, Applicant notes it is well established law that *an inherent characteristic must be necessarily present.*

> To establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is *necessarily present* in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.' In re Robertson,
>
> 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999), (emphasis added).

Though Breel et al. teaches that the four hybrids, when pulsed with an exogenous antigen, (KLH), can present this non tumor antigen to keyhole limpet hemocyanin (KLH) primed T cell, one of ordinary skill would not recognize that it would *necessarily follow* that the 4 hybrids acquired the physiological ability to induce an anti-tumor response when provided to a patient causing a reduction of the number of tumor cells in said patient as required by the claims, a property presently attributed to dendritic cells.

Breel et al. provides no data or information regarding the ability of the 4 referenced hybrids to induce an anti-tumor response when provided to a patient causing a reduction of the number of tumor cells in said patient as required by the claims. Therefore, in the

absence of more specific information describing the antigen processing properties of any of these 4 referenced hybrids, it *does not necessarily follow* that the method described by Breel et al. results in a hybrid which inherently possesses the required functional limitations of the instant claims, including activation of naive T cells.

In light of the above amendments and remarks demonstrating that the methods taught by Breel et al. produce hybrids that are patentably distinct from the hybrids produced by the instantly claimed methods, Applicant submits that Breel et al. is not an anticipatory reference. Accordingly, Applicants respectfully request reconsideration and withdrawal of the instant rejection. Claims 10, 13, 14, 18, and 20 have been canceled by Applicant without prejudice, rendering the rejection of these claims moot.

CONCLUSION

In view of Applicants' amendments to the claims and the foregoing Remarks, it is respectfully submitted that the present application is in condition for allowance. Should the Examiner have any remaining concerns which might prevent the prompt allowance of the application, the Examiner is respectfully invited to contact the undersigned at the telephone number appearing below.

Respectfully submitted,

Date: December 27, 2007

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The tetraspanin CD9 mediates lateral association of MHC class II molecules on the dendritic cell surface

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Communicated by Mark M. Davis, Stanford University School of Medicine, Stanford, CA, November 1, 2006 (received for review April 12, 2006)

We have found that MHC class II (MHC II) molecules exhibit a distinctive organization on the dendritic cell (DC) plasma membrane. Both in DC lysates and on the surface of living cells, I-A and I-E molecules engaged in lateral interactions not observed on other antigen-presenting cells such as B blasts. Because DCs and B blasts express MHC II at comparable surface densities, the interaction was not due to simple mass action. Instead, it reflected the selective expression of the tetraspanin CD9 at the DC surface. I-A and I-E molecules coprecipitated with each other and with CD9. The association of heterologous MHC II molecules was abrogated in DCs from CD9$^{-/-}$ mice. Conversely, expression of exogenous CD9 in B cells induced MHC II interactions. CD9 is thus necessary for the association of heterologous MHC II, a specialization that would facilitate the formation of MHC II multimers expected to enhance T cell receptor stimulation by DCs.

antigen presentation | major histocompatibility complex class II | B cell | CD81 | costimulatory molecules

The initiation of adaptive immunity requires presentation of antigens by dendritic cells (DCs) to naïve T lymphocytes (1). Why DCs are more efficient at stimulating naïve T cells than other antigen-presenting cells (APCs) remains incompletely understood. DC specializations that may contribute include the expression of high surface levels of MHC class II (MHC II) and costimulatory molecules, their regulated patterns of maturation and migration that coordinate their abilities to present antigens acquired in the periphery after arrival in lymphoid organs, their elaboration of long membrane projections that facilitate T cell contacts, and their secretion of an array of immunostimulatory cytokines (2). It seems likely, however, that other features remain to be discovered.

Upon antigen-specific contact between APC and CD4$^+$ T cells, molecular reorganizations take place at the surfaces of both cells, including polarization of T cell receptor (TCR) and MHC II to the center of the contact site (3). Recently, MHC II enrichment at T cell contact site was shown to result from LFA-1–ligand interactions and to require the APC actin cytoskeleton (4), suggesting that the APC may not be a passive partner in T cell interactions. Further evidence that T cell-dependent, TCR- or adhesion molecule-driven MHC II recruitment may not fully explain T cell responses is suggested by the fact that T cells can respond to single ligands (5, 6), whereas T cell activation requires TCR cross-linking, possibly induced by MHC II multimers (7, 8). Previous work suggested that MHC II on DCs exhibits a nonrandom distribution at least at the level of immunofluorescence (IF) (9, 10). Such preexisting clusters could facilitate TCR ligation and cross-linking. Although DCs are well known to regulate the intracellular transport of their MHC II molecules (11), little is known about MHC II dynamics at the DC plasma membrane (PM).

MHC II molecules have been reported to cluster with each other and with MHC class I (12, 13); "superdimers" were even observed in the original three-dimensional crystal structure of human MHC II (14). A few reports documenting surface MHC II dimer of dimers have followed, (15–17) but neither the significance nor specificity of these interactions has been established nor has their formation in physiological settings been demonstrated.

MHC II molecules have also been reported to interact with tetraspanins, "master organizers" of the cell surface (18). Several groups have reported interactions of MHC II with numerous tetraspanin family members in whole cell lysates of APCs (19–24). Coordinated interactions with specific tetraspanins at intracellular or PM locations have been proposed to be involved in MHC II distribution and function (25). An Ab to clustered MHC II (26) was found to coprecipitate several tetraspanins in APCs (27).

Conceivably, surface features that differentiate DCs from other potent APCs such as B cells may contribute to the DC's enhanced ability for naïve T cell stimulation. CD9 is one such differentiating feature, a tetraspanin that is selectively expressed in DCs and facilitates the association of heterologous MHC II molecules.

Results

Expression and Distribution of MHC II and Costimulatory Molecules on DCs and B Blasts. The enhanced efficiency of antigen presentation by DCs may in part simply reflect a higher level of expression of MHC and costimulatory molecules as compared with other APCs. To test this, we quantified the expression of MHC II and costimulatory molecules on mature bone marrow (BM)-derived DCs relative to B blasts, which also express high numbers of MHC II molecules. Quantitative flow cytometry was used, calibrated with fluorescence standards, to measure the numbers of MHC II (I-A or I-E), CD80, and CD86 molecules present on both cell types isolated from C57/B6 (I-A^b) or B10.BR (I-E^k) mice. Although the exact number of copies of a surface molecule cannot reliably be determined with the use of bivalent Ab, a reasonable and internally consistent approximation is possible.

Despite considerable cell to cell variation, it was clear that mature DCs expressed more of all three surface proteins (Table 1). In the case of MHC II, DCs had four to five times more than B blasts. Far greater differences were seen when expression of costimulatory molecules was compared. DCs expressed >10-fold more CD86 and CD80 than B blasts. Although earlier studies reported the numbers of MHC II molecules on the surface of human DCs severalfold higher (28, 29), the fact that our numbers are lower might reflect our having used a different species and Ab that were specific for only a single MHC II allele. In addition, we used directly conjugated primary Ab (molar ratio of fluor to IgG of 1:1), permitting stoichiometric comparisons with fluorescent beads and thus avoid-

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Abbreviations: DC, dendritic cell; MHC II, MHC class II; APC, antigen-presenting cell; TCR, T cell receptor; IP, immunoprecipitation; PM, plasma membrane; IF, immunofluorescence; BM, bone marrow; I-E beads, anti-I-E-coated beads.

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Table 1. Expression and surface density of MHC II products, CD80, and CD86 by DCs and B blasts

Antibody	Mouse strain	Molecules per cell* DC†	Molecules per cell* B†	Molecules per μm^2 DC	Molecules per μm^2 B
I-A^b AF6 120.1	C57/B6	111 ± 36	28 ± 9.0	77.9	115.7
I-A^b TIB120‡	C57/B6	191 ± 47	71 ± 43	134.1	290.5
I-E^k TIB120‡	B10.BR	80 ± 21	16 ± 1.5	56.4	63.8
I-E^k 14.4.4S	B10.BR	124 ± 21	30 ± 4.2	87.3	124.6
I-Ak 11-5.2	B10.BR	188 ± 77	39 ± 3.4	132.1	159.7
CD86 GL1	C57/B6, B10.BR	208 ± 43	16 ± 1.6	146.3	67.3
CD80 16-10A1	C57/B6, B10.BR	132 ± 20	1.6 ± 0.7	93.1	6.5
CD40 3/23	C57/B6, B10.BR	17 ± 1.8	2.1 ± 1.0	11.9	8.5
CD11a	C57/B6, B10.BR	27 ± 7.6	9.7 ± 4.8	19.2	39.7
Isotype control	C57/B6, B10.BR	3.0 ± 1.6	0.9 ± 0.3	2.1	3.8
Cell volume		965 μm^3	254 μm^3		
Cell surface area		1,421 μm^2	243 μm^2		

Mature DCs or 24-hr B blasts were labeled at saturating concentrations with the indicated PE-conjugated Ab and processed for flow cytometry. Fluorescence standards were used to estimate the number of molecules per cell on mature DCs or fully blasted B cells by using beads with known quantities of fluors to perform a linear regression analysis. Numbers of molecules ± standard deviation (× 10^3) are listed for each Ab and mouse strain (columns 3 and 4). Pellets of mature DC or 24-hr B blasts were prepared for electron microscopy, serial sections were cut, and cell volume and surface area were calculated using the disector method. Numbers of molecules per square micron were obtained by dividing total number of molecules per cell by surface area in square microns (columns 5 and 6).

*Values are stated as numbers of molecules × 10^3.

†Gated on CD11c + or B220 + populations, respectively.

‡The mAb TIB 120 (M5/114.15.2) is specific for I-A^b, I-A^q, I-A^d, I-E^d, and I-E^k.

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ing possible signal amplification by using fluorescent second Ab, as in previous studies.

We normalized these numbers to account for differences in cell size. Mean surface area and volume for mature DCs and B blasts were determined from serial sections by electron microscopy and stereology. The average volume and surface area of DCs were four and six times greater than B blasts, respectively (Table 1), so that the effective density of MHC II molecules on the DC surface was ≈1.3 times lower than on B cell blasts. The density of costimulatory molecules, however, remained higher on DCs (2- to 14-fold), with the exception of CD40 present at comparable levels.

Although the density of MHC II was higher in B blasts, previous work suggested that DCs might organize their MHC II and CD86 in small patches on the PM (10). To determine whether this suggestion was unique to DCs, we visualized the distribution of these molecules in both cell types by IF. As before, the heterogeneous distribution of MHC II and CD86 was best visualized in DCs at an "intermediate" stage of maturation, i.e., before a fully mature "dendritic" phenotype was established (Fig. 1*A*). In contrast, in B blasts, MHC II and CD86 molecules always appeared homogeneously distributed (Fig. 1*B*). Morphological differences in these images reflect the fact that DCs spread when they attach to coverslips, whereas the B blasts remain spherical.

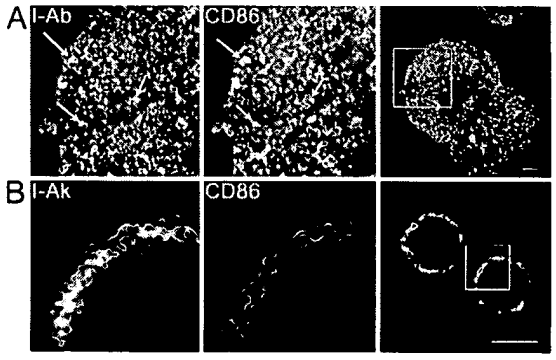

Fig. 1. DCs, but not B cells, cluster surface MHC II and CD86. DC (*A*) were matured for 6–12 h, after which they or 24-h B blasts (*B*) were prepared for IF microscopy. Confocal images of a single plane are shown. (Scale bar, 5 μm.) Areas boxed in the right, merged panels are shown at higher magnification in individual colors in the left (MHC II, green) and middle (CD86, red) panels. Arrows show examples of PM regions with clustering of both molecules.

Lateral Interaction of Heterologous MHC II Molecules in Live DCs. To confirm that the observed patchiness reflected the existence of lateral interactions that did not occur in B blasts, we developed an assay to determine whether cross-linking one species of MHC II resulted in the coclustering of a second MHC II species in live cells, avoiding possible artifacts involving Ab-induced clustering or cell fixation and permeabilization. Polystyrene latex beads were coated with anti-MHC II Ab specific for either I-A or I-E, allowed to interact with DCs or B cells expressing GFP-tagged I-E α chain, and then assayed by confocal microscopy to determine whether I-E-GFP clustered at sites of bead attachment. Under the conditions used, the beads largely remained at the surface of both DCs and B cells. The beads' regular geometry and 1:1 stoichiometry of GFP fluorescence to MHC II facilitated quantitation.

After a short incubation with beads at 37°C, anti-I-E-coated beads (I-E beads), as expected, efficiently clustered I-E-GFP on both cell types (Fig. 2 *A* and *B Upper*), with brighter GFP fluorescence at sites of bead attachment than elsewhere on the cell. Unattached beads exhibited negligible autofluorescence. On DCs, the fluorescence was typically circumferential around the bead, not because the beads had been internalized but rather because they had adhered to the upper surface of flattened cells, with confocal images taken in a single X-Y plane. On B blasts, beads were generally bound to the cell margins, thus GFP fluorescence was limited in distribution to the point of attachment.

More interesting were the results obtained by using I-A beads. Despite weaker GFP fluorescence, I-A beads clustered I-E-GFP on DCs, but no such clustering was observed for I-A beads on B blasts (Fig. 2 *A* and *B Lower*, arrow). These results are reminiscent of

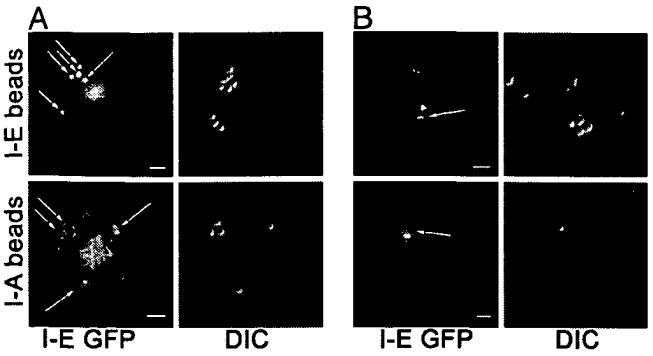

Fig. 2. DCs, but not B cells, recruit irrelevant MHC II to site of bead contact. After adhering I-E-GFP-expressing DCs (*A*) or B blasts (*B*) to coverslips, Ab-coated beads were added and incubated with cells at 37°C for 10 min, followed by fixation and fluorescence microscopy. (*Upper*) I-E beads. (*Lower*) I-A beads. (Scale bars, 5 μm.) Confocal images of a single plane are shown (*Left*), and DIC images show bead position (*Right*). Arrows show sites of bead contact and/or I-E enrichment.

studies reporting glycolipid raft-mediated clustering of irrelevant MHC II molecules at the synapse of T cells with a B cell line (30).

To ensure that the results did not simply reflect a nonspecific accumulation of membrane at the contact sites between beads and DCs or B blasts, we performed control experiments using uncoated beads, beads coated with irrelevant Ab (data not shown), or I-E beads bound to cells expressing CD86-cyan fluorescent protein (in addition to I-E-yellow fluorescent protein) as an unrelated PM protein. I-E beads failed to cluster CD86-cyan fluorescent protein on either DCs or B cell blasts, even at sites where there was abundant clustering of I-E-yellow fluorescent protein [see supporting information (SI) Fig. 7, arrows]. In this image, I-E-yellow fluorescent protein has been recruited to the beads to such an extent that the I-E-yellow fluorescent protein on the remainder of the cell has been depleted, making the overall fluorescence comparatively low. We quantified these data by counting beads in contact with a GFP-expressing cell, scoring the frequency at which the bound beads induced morphologically identifiable clustering (Fig. 3*A*). Clustering of I-E by I-E-beads (specific clustering) occurred to similar extents in both DCs and B blasts. The frequency of I-E-GFP clustering by I-A beads (nonspecific clustering), however, was 77% that of specific clustering in DCs, whereas in B blasts it was <10%. In those rare instances in which I-E-GFP clustering

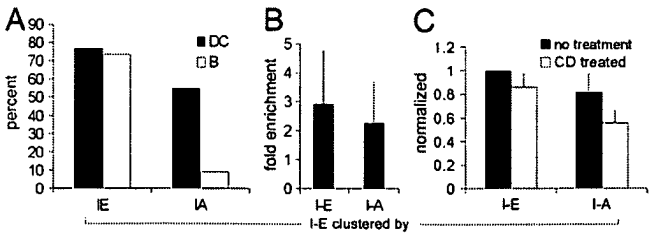

Fig. 3. Quantitation of bead-associated fluorescence. (*A*) DC vs. B blast bead-induced clustering. The percentage of beads in contact with I-E-GFP-expressing APCs with enrichment of I-E upon incubation with I-A or I-E beads was quantified. DCs, filled bars; B blasts, open bars. (*B*) DC-specific vs. nonspecific clustering. Enrichment of I-E fluorescence at sites of bead contact vs. elsewhere on the same cell were quantified by fluorescence intensity (see *Materials and Methods*); fold enrichment of I-E clustering by I-E or I-A beads is shown. (*C*) Effect of methyl-β-cyclodextrin treatment. After adhering DCs to coverslips, they were treated with 30 mM methyl-β-cyclodextrin, beads were added and processed as above, and fold enrichment of I-E by I-E and I-A beads was measured. Fold enrichment without (filled bars) and with (open bars) cyclodextrin treatment was normalized to without treatment.

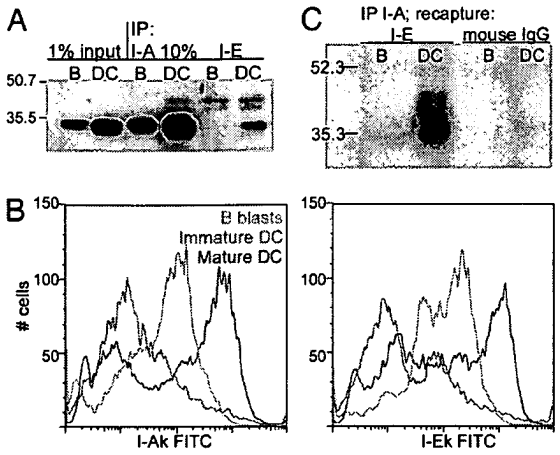

Fig. 4. MHC II I-E associates with I-A in DC but not in B blasts. (*A*) Immunoprecipitations of 1% CHAPS lysates of B blasts and DCs were followed by SDS/PAGE and Western blotting for I-A. B blast lanes contain 2-fold the protein concentration of DC lanes to correct for amounts of MHC II. Lanes 1 and 2 illustrate 1% input of lysates used for IP. I-A lanes, IP with mAb 10-2.16; I-E lanes, IP with mAb 14.4.4S. (*B*) I-A (*Left*) and I-E (*Right*) expression on B blasts (green lines), immature DC (red lines), and mature DC (blue lines) by FACS. (*C*) DCs (lanes 2 and 4) or B blasts (lanes 1 and 3) were metabolically labeled with ^{35}S, and after an overnight chase, I-A molecules were immunoprecipitated with mAb 10-2.16. Material was released from the beads by treating with Triton X-100, and I-E was recaptured from the IP with mAb 14.4.4S (lanes 1 and 2) or control IgGs (lanes 3 and 4), followed by SDS/PAGE and fluorography.

occurred in B cells, it was barely detectable. Indeed, what appeared as clustering in 2 out of 22 cells scored as positive might have reflected I-E-GFP fluorescence in the Golgi/recycling endosome region of the B cell cytoplasm, which was occasionally close to the PM (Fig. 2*B*).

The clustering results were further analyzed by measuring relative fluorescence at sites of bead contact vs. nonbead regions of the PM of DCs. Quantitation revealed a 3-fold enrichment of I-E-GFP fluorescence by I-E beads and 2.25-fold by the I-A beads; these were not statistically different (Fig. 3*B*). Thus, there appeared to be a significant degree of lateral association of I-A and I-E molecules on the PM of DCs. Because a fraction of MHC II has been observed to associate with glycolipid-cholesterol-enriched lipid microdomains (rafts) in B cells and in DCs (31–33), and because complexes of MHC II with other molecules including the tetraspan CD9 (see below) have been reported to be slightly enriched in lipid rafts (24), we wondered whether these microdomains might be involved in corecruitment of I-A and I-E molecules using I-E beads. To test this possibility, I-E-GFP-expressing DCs and B blasts were treated with methyl-β-cyclodextrin to deplete cholesterol and then challenged with I-E or I-A beads. Although the amount of I-A corecruitment was decreased in the treated cells, it decreased in direct proportion to a decrease in I-E clustering (Fig. 3*C*). These results, while not excluding a role for rafts, provided no evidence in favor of cholesterol-dependent lipid microdomains as responsible for the recruitment of irrelevant MHC II.

I-E and I-A Molecules Can Be Coimmunoprecipitated from DC but Not B Cell Lysates. Because glycolipid rafts did not appear to mediate the association between I-A and I-E molecules, we asked whether protein–protein interactions might be responsible. Mature DCs and B blasts were lysed in CHAPS and subjected to immunoprecipitation (IP) by using I-A or I-E Ab. Twice as much total protein was used in B blast as in DC lanes to adjust for higher levels of expression of MHC II in the DCs (Table 1). The precipitates were then probed by Western blotting by using an Ab to I-A (available reagents for I-E were not useful for Western blotting). Input lanes

(Fig. 4A, left lanes) and I-A IPs (center lanes) confirmed that comparable amounts of I-A were present in normalized B and DC lysates. I-E IPs, however, contained considerably more I-A in DCs than in B blasts (right lanes in Fig. 4A). This difference did not reflect a disparity in relative levels of I-A and I-E expression because both cell types express both alleles at similar ratios (Fig. 4B). As a control, we used Ab to CD86, another molecule expressed almost exclusively at the DC or B cell surface; only negligible amounts of I-A were detected as coprecipitating with CD86 in either cell type (data not shown).

The biochemical association of I-E with I-A could also be shown via sequential IP following metabolic labeling. Mature DCs and B blasts were labeled with [^{35}S]Met/Cys and, after an overnight chase, I-A molecules were immunoprecipitated from CHAPS lysates. The presence of I-E was determined by recapture of I-E from the I-A IP, followed by SDS/PAGE and fluorography. A band of the correct molecular weight was present in the DC lysate (Fig. 4C, lane 2), with only trace amounts in the case of B blasts (lane 1). No band was recaptured by using a control Ig (lanes 3 and 4).

Because CHAPs is well known to preserve protein–protein interactions, we repeated these experiments using Triton X-100 lysates, another nonionic detergent that more often disrupts such interactions. I-A could not be detected by Western blotting as having coprecipitated with I-E in Triton X-100 lysates from either B cells or DCs (data not shown), consistent with the possibility that protein–protein interactions were responsible.

The DC-Enriched Tetraspanin CD9 Forms a Complex with MHC II Molecules. An association between I-A and I-E in DCs might reflect a direct or indirect interaction among dissimilar MHC II molecules. We sought to test whether adaptor proteins were involved, considering members of the tetraspanin family and given the many previous reports of their interactions with MHC II products. Most previous work, however, focused on potential interactions in B cell lines, concentrating on tetraspanins that localized predominantly to MHC II-positive lysosomal compartments as opposed to the DC PM (19–22). In both human B lymphoblastoid cell lines and monocyte-derived DCs, cell fractionation has shown that the MHC II-associated tetraspanins CD63 and CD82 are predominantly localized to lysosomes (23, 25, 34). In contrast, in human monocyte-derived DCs, CD9, CD53, and CD81 are largely nonlysosomal and probably mostly at the PM (25, 34). We screened mouse BM-derived DCs and B blasts for their expression and localization of two predominantly surface tetraspanins, CD9 and CD81. Although CD81 was highly expressed on the surface of B blasts, it was barely detectable at the DC surface by FACS (Fig. 5A). In contrast, CD9 was found on the PM of DCs, enhanced slightly during maturation, at levels >10-fold higher than B blasts, (Fig. 5A). Although the roles of some tetraspanins may be overlapping or redundant (18), we reasoned that because CD9 was expressed at such high levels on DCs, and because at least one other tetraspanin (CD81) was present at much lower levels, CD9 represented the best candidate for facilitating the observed MHC II interactions.

By IF microscopy of fixed and permeabilized cells, CD81 expression on the surface of DCs was found to be low and moreover localized largely to intracellular compartments (Fig. 5B *Top*). In contrast, CD9 was observed predominately at the PM where it exhibited a patchy distribution, similar to MHC II (Fig. 5B *Middle*). In fact, it appeared that the distributions of surface CD9 and MHC II at least partially overlapped when live DCs were labeled with Ab to both markers (Fig. 5B *Bottom*).

We next asked whether either or both CD9 and CD81 would co-IP MHC II molecules. After IPs of CHAPS lysates using anti-CD9 or anti-CD81 Ab, the precipitates were probed for the presence of I-A molecules by Western blot analysis (Fig. 5C). As shown in Fig. 5C *Left*, far more I-A was present in CD9 IPs from DCs than from B blasts. In the case of CD81, however, similar amounts of I-A were coprecipitated from DCs and B blasts (*Right*).

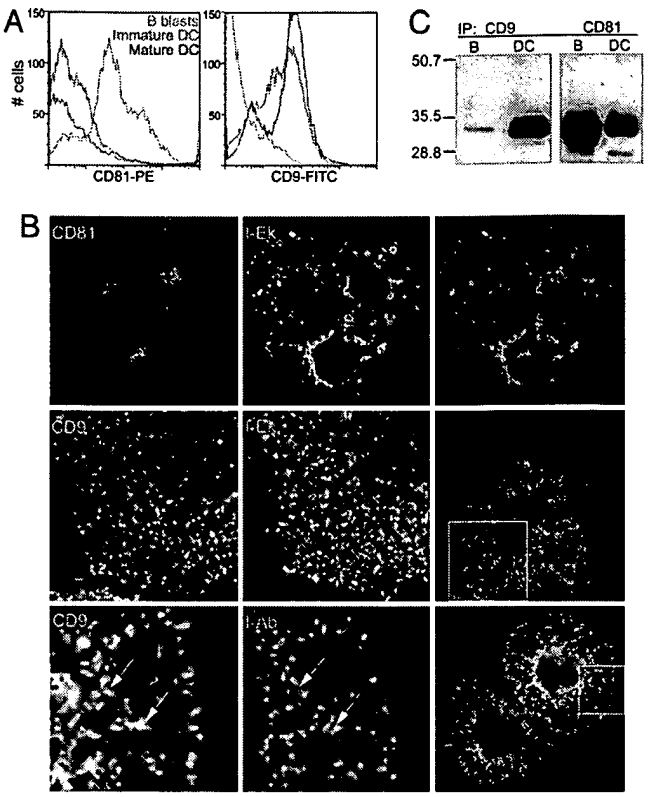

Fig. 5. Selective association of CD9 with MHC II in DCs as compared with B blasts. (*A*) Expression of selected tetraspanin molecules on APCs. Green lines, B blasts; red lines, immature DC; blue lines, mature DC. (*Left*) CD81. (*Right*) CD9. (*B*) IF of DCs labeled with I-Ek (red) and the following: (*Top*) CD81 (green, permeabilized); (*Middle*) CD9 (green, permeabilized); and (*Bottom*) CD9 (green, live, nonpermeabilized labeling on ice). Boxed areas on merged right panels are shown at higher magnification in individual colors. Arrows indicate examples of partial CD9 and MHC II surface colocalization. Confocal images of a single plane are shown. (*C*) Immunoprecipitations and Western blotting for I-A were carried out as in Fig. 4A. CD9 IPs (lanes 1 and 2) as compared with CD81 IPs (lanes 3 and 4) from B blasts and DCs, respectively.

CD9 Is Necessary for I-A/I-E Associations. To test whether the I-A/I-E association requires CD9, BM-derived DCs were cultured from CD9$^{-/-}$ mice (35) and tested for I-A/I-E association by using I-A or I-E beads to cluster retrovirally expressed I-E-GFP. In these experiments, the fold enrichment of both specific and nonspecific clustering at sites of bead contact was slightly lower (e.g., Fig. 3) because of a lower level of I-E-GFP expression in the cells used. Nevertheless, after quantitation of fluorescence at sites of bead attachment (performed as in Fig. 3B), it was evident that, in the absence of CD9, no enrichment of heterologous MHC II occurred (Fig. 6A). Thus, CD9 was necessary for heterologous MHC II association in this assay.

To confirm independently that CD9 was responsible for mediating the interaction between I-A and I-E, we performed a gain-of-function experiment, using B cells as naturally occurring CD9-negative cells, and asked whether I-A/I-E interactions could be induced by expression of exogenous CD9. A20 B lymphoma cells, in which, as in primary blasts, I-E association with I-A was found to be negligible, were transfected with the CD9 cDNA (36), and stable lines were created (Fig. 6B). Transfectants expressed highly variable levels of CD9, and only a small minority of cells expressed levels of CD9 comparable to DCs (Fig. 6B). As shown in Fig. 6C, upon exogenous expression of CD9, I-E IPs were observed to contain I-A by Western blotting (Fig. 6C, I-E lanes; bands corresponding to MHC II and IgG are indicated with arrows).

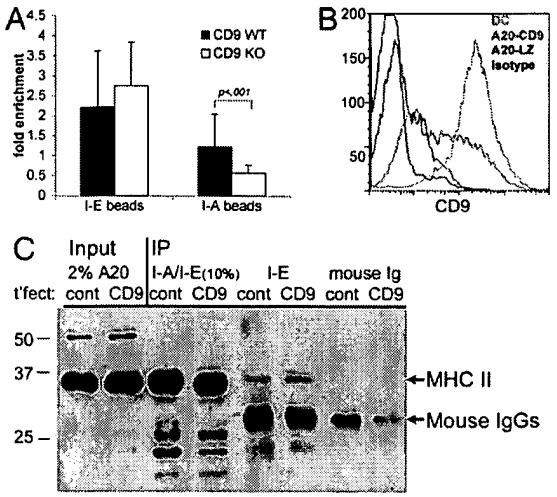

Fig. 6. CD9 is necessary for I-A/I-E clustering. (*A*) DCs raised from the BM of CD9 WT vs. knockout mice and retrovirally transduced with I-Eα-GFP were adhered to coverslips and allowed to interact with I-E- or I-A-coated beads as in Fig. 2. Fluorescence at sites of bead adherence was quantified as described in Fig. 3. (*B*) A20 B lymphoma cells were transfected with CD9 cDNA (red line) or lacZ as a control (blue line). DCs are shown for comparison (green line). Black line, isotype control. (*C*) CHAPS lysates (1%) of stable cells lines (A20 cells transfected with CD9, lane 6, or a control plasmid, cont., lane 5) were immunoprecipitated with Ab to I-A or I-E as in Fig. 4, and I-A was visualized by Western blotting. Total MHC II was precipitated with TIB120, specific for both I-A and I-E in these cells (lanes 3 and 4). IP with an irrelevant Ab is shown in lanes 7 and 8. Arrows indicate MHC II and mouse IgG bands.

Relatively little I-A was detected by blotting I-E IPs from the CD9-negative control. The exogenous expression of CD9 is thus sufficient, given the presence of other tetraspanins, for inducing I-A/I-E association in an otherwise CD9-negative B cell line.

Discussion

We compared DCs to B blasts to seek differences possibly responsible for DCs' superior antigen presentation ability. As expected, DCs express higher numbers of MHC II and costimulatory molecules than B blasts. It is surprising, however, that B blasts, known to be less efficient at T cell stimulation, actually achieve a higher density of MHC II molecules per unit of PM. These results are consistent with earlier findings that B cells require 10-fold more MHC-peptide complexes than DCs to effect the same level of T cell stimulation (37). Thus, although DCs are able to deliver a more potent stimulus to T cells, they do so while expressing a lower surface density of TCR ligand. Although this may in part be explained by DCs' higher density of CD86 and CD80, we propose that surface organization of MHC II may also play a role in facilitating antigen presentation. The patchy distribution of DC surface molecules presents the T cell with preclustered "packets" of MHC II I-A and I-E, potentiating T cell activation by facilitating TCR clustering and signaling (7, 38). The DC is thus able to distribute TCR ligands over a larger surface area, allowing for simultaneous interaction with multiple T cells.

Even with clustered TCR ligand, the next challenge is finding the agonist TCR ligands in a large excess of self-peptide complexes. The unexpected finding of association between heterologous MHC II molecules in DCs provided us with a possible mechanism for TCR clustering. The pseudodimer model proposes interaction of two TCRs with adjacent MHC II-peptide complexes, one loaded with an endogenous, the other with an agonist, peptide, the two receptor-ligand pairs bridged by a CD4 molecule (5, 39). With homogeneously distributed, freely diffusing MHC II molecules, there would be one MHC II molecule per ≈9,200 nm^2 of surface area, placing each an average of 96 nm away from its nearest neighbor, such that the probability of forming the pseudodimer would appear to be extremely low. Although lateral diffusion may compensate, tethering similar or dissimilar MHC II molecules together increases the likelihood that a CD4 molecule can bind a neighboring MHC II molecule. Although we did not measure the association of homologous MHC II molecules, we surmise that I-E/I-E pairs are just as likely as I-E/I-A ones. From the standpoint of a role in TCR stimulation, the existence of homodimers would be just as important.

Our results further indicate that the discontinuous distribution of MHC II on the DC surface and the lateral interaction of heterologous MHC II products are mediated by association with members of the tetraspanin family. Although association of MHC II with tetraspanins has been observed, previous work has yet to assign a physiologically relevant function to a specific tetraspanin in APCs. We have provided direct evidence that a major surface tetraspanin on DCs, CD9, plays an essential role in physically tethering dissimilar MHC II molecules together in a fashion that distinguishes DCs from other APCs. We also documented the MHC II/CD9 association in both gain-of-function and loss-of-function experiments, focusing on CD9 as the tetraspanin present in our compartment of interest, the DC PM.

Our results are consistent with but also quite distinct from recent work of Kropshofer *et al.* (27). In human DCs, an Ab (CDw78) thought to detect "clustered" MHC II was found to coprecipitate the tetraspanins CD81 and CD9, data interpreted to suggest the existence of a tetraspanin domain either at the DC PM and/or intracellularly (27). This pool of MHC II molecules also contained some that were loaded with the invariant chain-derived peptide CLIP, which could be found at T cell interaction sites where it may act to modulate the T cell response to produce Th2 lymphocytes (40). Possibly any tetraspanin localized to the PM could mediate the observed MHC II interactions, but in DCs it is CD9 that is present at high levels and carries out this role. Thus, at a minimum, "tetraspanin domains" on the DC PM would reflect the ability of CD9 to facilitate the lateral interactions of individual MHC II molecules.

Although our data provide no evidence for a role for glycolipid rafts in mediating the observed heterologous MHC II associations, they do not exclude the possibility that these microdomains are involved. The presence of irrelevant MHC II molecules in the immunological synapse (30, 41) is intriguing and may or may not be related to the findings presented here. The MHC II interactions we observe are present before T cell contact and the extensive membrane reorganizations that happen upon antigen-specific encounter between APC and T cell.

Although the question of the function of CD9 in antigen presentation is beyond the scope of this paper, it is interesting to note that ligation of CD9 (by pregnancy-specific glycoprotein 17) inhibits the proliferation of naïve T cells, suggesting a role for CD9 in T cell responses (42).

DCs are not the only cells known to express CD9: defined subsets of B cells have been reported to express CD9 (43), and interestingly it is the same CD9+ subset which has been shown to be more efficient at stimulating naive CD4$^+$ T cells (44). It is possible that the improved T cell stimulatory capacity of CD9-expressing B cells is due to their ability to phenocopy the DC PM, at least with respect to the lateral association of MHC II. With the finding that CD9 is capable of inducing interactions of MHC II in living cells, we have defined one of the very few discrete biochemical roles yet described for this or any other member of the tetraspanin family. It will now be possible to interrogate this interaction by using a variety of approaches to define the larger functional significance of this property of CD9 and MHC II.

Methods

For additional details, see *SI Methods*.

Mice and Cells. DC were grown as described (45) from BM progenitors of B10/BR, C57/Black6, (Jackson Labs, Bar Harbor, ME), or CD9 WT vs. knockout mice (35). B blasts were prepared from B10.BR or C57/Black6 spleens as described (46). A20 B lymphoma cells were transfected with the CD9 cDNA in pEF6/V5-His [(36); gift of G. Dveksler, Uniformed Services University of the Health Sciences, Bethesda, MD] or a control plasmid, lacZ-pEF6/V5-His (Invitrogen, Carlsbad, CA), and stable lines created by selection with blasticidin (Sigma, St. Louis, MO), at 2 μg/ml.

Antibodies and IF. If was done as described (9) by using the following monoclonal Ab: KH74 (I-A^b), GL1 (CD86), AMS 32.1 (I-A^d), and CD81 (TAPA-1) from BD-PharMingen (San Diego, CA); TIB120 (M5/114.15.2, I-A/I-E) and 14.4.4S (I-E) from ATCC (Manassas, VA); CD9 (KMC8.8; BD-PharMingen and ATCC); and 10-2.16 (I-A^k) and Y3P (I-A) (gifts of the Bottomly/Janeway laboratory, Yale University, New Haven, CT). Polyclonal rabbit anti-I-Aβ were also used (Rivoli and Thorax) (10, 47).

For cyclodextrin treatment, DCs were plated as usual on coverslips, washed in serum-free RPMI medium 1640, and then incubated with 30 mM methyl-β-cyclodextrin (Sigma) for 10 min at 20°C, followed by three RPMI medium 1640 washes, after which they were used in the bead assay.

Cell Size Calculations and Stereology. B cells and DCs were fixed, dehydrated, and embedded in Epon, and serial sections were cut and examined by electron microscopy. Stereology was used to estimate the cell size by using the dissector method.

Bead Assays. Ab were bound to Dynabeads protein A (Dynal, Great Neck, NY) per manufacturer instructions. Cells were plated as for IF, then 1–5 $\times$ 10^5 beads were added to cells in complete medium and centrifuged for 2 min at 70 $\times$ g, incubated at 37°C for 10 min, fixed with 4% paraformaldehyde, and visualized as above. Quantitation of conflated confocal x-z sections was done by using the following formula: fold enrichment = (bead fluorescence − autofluorescence of bead not on GFP-expressing cell − background)/[bead area cell fluorescence (next to bead) − background].

FACS Analysis. Flow cytometry was done with a FACSCalibur and CellQuest software (Becton Dickinson, Franklin Lakes, NJ) for acquisition and FloJo (TreeStar, Ashland, OR) was used for analysis. Numbers of surface molecules on CD11c+ or B220+ populations were quantified by using QuantiBrite phycoerythrin beads (BD Biosciences, San Jose, CA) as per manufacturer instructions. Events (10^4) were collected in each of three experiments.

Statistical Analysis. The two-tailed Student t test was used to determine statistical significance.

Biochemical Assays. CHAPS lysates (1%) of mature DC or B blasts after 24 h of culture were precleared with protein G Sepharose beads (Amersham Pharmacia, Piscataway, NJ), followed by 4–15 h IP of 1 mg lysate with 10 μg mAb bound to protein G Sepharose, and analyzed by 12 or 15% SDS/PAGE (Bio-Rad, Hercules, CA), transferred to nitrocellulose membrane (Schleicher and Schuell, Florham Park, NJ), Western blotted, and visualized by SuperSignal Femto chemiluminescence reagent (Pierce, Rockford, IL).

Metabolic Labeling and Immunoprecipitation/Recapture. Cells (DC at day 5, 6 h after maturation stimulus and B blasts after 3 h LPS stimulation) were washed in Cys/Met-free media and then pulsed with [^{35}S]Cys/Met (Amersham Biosciences) for 1 h at 37°C, 5% CO_2. After two PBS washes, cells were cultured overnight in complete media. After first IP, material was eluted from beads with 1% Triton X-100 in 100 mM Tris·HCl (pH 7.4). After SDS/PAGE, gel was soaked in 1 M salicylic acid (Sigma) for 1 h before processing for fluorography.

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